

# Glacial and postglacial species divergence and dispersal of European trickle midges (Diptera: Thaumaleidae)

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## Abstract

Pleistocene glaciations have greatly influenced the current distribution and diversity of aquatic and terrestrial species in Europe. We studied the phylogeography and the genetic structure of European trickle midges (Diptera: Thaumaleidae). This family is restricted to hygropetric zones with the genera *Thaumalea*, *Protothaumalea* and *Androposopa* occurring in Europe, including both microendemisms and species widely distributed across the continent. A 658-bp fragment of the mitochondrial CO1 and a 563-bp fragment of the nuclear Wingless-gene from 56 individuals belonging to 14 species were sequenced and analyzed. *Androposopa* is monophyletic and sister group to a broad *Thaumalea* clade which includes *Protothaumalea*. All species for which multiple populations were available are monophyletic, while the previously suggested hypothesis of species groups within *Thaumalea* is not supported. To understand the influence of glacial dynamics on Thaumaleidae evolution, we investigate four different scenarios for species divergences, testing different periods for within-species population splits. The results suggest different evolutionary histories for different species. For *Thaumalea testacea*, we found evidence for survival in multiple Alpine refugia throughout the glacial maxima. On the other hand, *T. bezzii* seems to have dispersed into Central Europe from the East Mediterranean area after the last glaciation. For the Faroe Islands populations of *T. veralli*, dispersal could have happened by air currents from Europe rather than by anthropogenic transport. Overall, our results show a wide range of dispersal patterns within an otherwise uniform group of organisms, opening new avenues for further studies in phylogeography and speciation.

## Key words

Biogeography, phylogeny, phylogeography, speciation, glacial refugia, spring organisms.

## 1. Introduction

In the Pleistocene, glacial movements influenced diversity and distribution of all organisms in Europe (HEWITT 1996), inducing fragmentation and population declines in many terrestrial and aquatic animals. Afterwards, many species experienced postglacial range expansions in the Holocene (KOTLIK et al. 2008; VILA et al. 2005). Previous studies suggest that colonization of Central Europe started from multiple refugial areas during interstadial, interglacial or postglacial periods (HEWITT 1996,

1999; SCHMITT & SEITZ 2001; WIDMER & LEXER 2001; PAULS et al. 2006; MALICKY 2006). Although the majority of studies focused on terrestrial animals, more attention has been recently devoted to freshwater invertebrates (e.g. PAULS et al. 2006; MACHER et al. 2015). For aquatic insects, historic isolation can be considered as the primary force shaping the contemporary genetic variation (BÁLINT et al. 2011; ALPT et al. 2012; THEISSINGER et al. 2012). In particular, organisms inhabiting springs and

seepages are scantily studied (BENKE et al. 2009; HEWITT 1996). Note that freshwater springs are different from other aquatic refugia, because they seem to have provided sustained refugia for aquatic invertebrates in Europe throughout glacial periods (UJVAROSI et al. 2010; MALICKY 2006). Recent studies by PAULS et al. (2006) and MACHER et al. (2015) showed that distribution and dispersal of aquatic organisms during the Pleistocene differed among species due to persistence in refugia and multiple glacial cycles.

Thaumaleidae are tightly associated with hygropetric freshwater zones, mostly found in proximity to springs. This small family of culicomorph Diptera includes approximately 200 species – about 80 occur in Europe – from eight genera with worldwide distribution. Larvae graze on algal mats and live hygropetrically, adapted to seepages with cool temperature, whereas adults do not feed and are rarely found far from the larval habitat (WAGNER 2002). Limited dispersal abilities, high habitat specificity and fragmented distributions make Thaumaleidae an ideal taxon for studying genetic diversification through geographic isolation in different refugia. However, few specimens are available presumably due to high specialization, small habitat size, and the short lifetime of adults. Thus, few studies address their evolution, biology and systematic and most of the literature is limited to basic taxonomic issues. Fortunately, the species-level classification of the European fauna appears relatively stable after revisions by MARTINOVSKÝ & ROZKOSNÝ (1976), VAILLANT (1977) and WAGNER (1995). Three genera, *Androposopa*, *Protothaumalea* and *Thaumalea*, are recognized, the latter comprising most of the described species (70; WAGNER 2002). WAGNER (2002) established an infrageneric species-group system in *Thaumalea* based on morphological characters of male genitalia.

Although it is generally believed that the Alps are a natural barrier, hybridization zone and origin of speciation for several organisms during and after the ice ages (HEWITT 1995; BRÄNDLE et al. 2007; GRAF et al. 2009), we do not have any information on glacial refugia or distribution and recolonization events for Thaumaleidae during interglacial or postglacial times. Depending on species dispersal and cold-tolerance capabilities, several species may have survived during times of glaciations in small extra-Mediterranean refugia, even in Central Europe (MALICKY 1983, 1988; PAULS et al. 2006), or dispersed postglacially. As some species of *Thaumalea* can withstand freezing temperatures at the egg stage (STERNBERG 1997), very small marginal refugia could have hosted permanent populations. However, postglacial dispersal in meltwater and subsequent fragmentation by warming or anthropogenic landscape changes could have caused disparate distribution patterns.

Due to the different periods, glacial and postglacial dispersal events could lead to different genetic signatures in separated populations. The present study aims at exploring the influence of glaciations on biogeography, speciation and intraspecific genetic divergences in Central Europe. Using a data set of Thaumaleidae spe-

cies and populations from Europe, we investigated the different glacial, interglacial and postglacial diversification scenarios to explore both the hypotheses of glacial fragmentation and postglacial dispersal events. We then discussed the implications of our phylogenetic structure for the taxonomy and systematics of the family.

## 2. Methods

### 2.1. Material used

We successfully obtained sequences from 56 specimens belonging to 14 species and 16 localities (see Supplementary Table S1). Other available material was either too old for obtaining sequences or deemed taxonomically too important (e.g. types, rarely collected species) to be extracted. Identifications and nomenclature were made according to WAGNER (2002), except that *Thaumalea cebbennica* Vaillant is recognized as a synonym of *T. truncata* Edwards in agreement with ANDERSEN et al. (2013), and *Androposopa larvata* is considered a potential species group (“*Androposopa* gr. *larvata*”) due to the high morphological variability among examined populations. Specimens were stored in 96% EtOH until DNA was extracted. Partial voucher specimens consisting of head, wings and abdomen are preserved in alcohol and will be stored at Science Museum Alexander König (ZFMK) in Bonn. Sequences for outgroup taxa were retrieved from GenBank (see Supplementary Table S1).

### 2.2. Laboratory procedures

Genomic DNA was extracted using DNeasy Blood & Tissue Kits according to the manufacturer’s protocol. We used polymerase chain reaction (PCR) to amplify mitochondrial cytochrome oxidase I (COI) and nuclear wingless (WG) in 25 µl reactions using 2 µM of the loci specific primer pair and 5 µl DNA templates. COI was amplified using primers from FOLMER et al. (1994), according to PCR programs described in SHOKRALLA et al. (2010). For amplification of the WG-gene, primers and PCR programs follow PAULS et al. (2008). Sequencing was carried out by GATC Ltd. Konstanz, (Germany) using the above-mentioned stated primers. ABI traces were aligned and manually checked using the program Geneious R6 (KEARSE et al. 2012). Only unambiguous sequences, verified by BLAST (ALTSCHUL et al. 1997), were included. Sequences with heterozygote double peaks were identified using the program Codoncode (WECKX et al. 2005); heterozygous signals were coded using IUPAC codes. Final sequences were aligned for each gene using MAFFT v7.017 (KATO et al. 2002). COI and WG alignments of 56 Thaumaleidae and outgroup specimens were exported and concatenated into a 1221 bp long alignment without gaps or ambiguities for subsequent analyses.

**Table 1.** Listing of calibration points and prior information for all specified nodes. Bounds were set according to previously set scenarios (Sc.1–4).

Node	Prior Distribution	Bound	Monophyletic constraint	Origin	Comment
Thaumaleidae	Log Normal	66–100 Ma	✓	Fossil	KOVALEK 1989
Culicidae	Log Normal	70.6–99.6 Ma	✗	Fossil	BORKENT & GRIMALDI 2004 POINAR et al. 2000
<i>Culex</i>	Gamma	33.9–55.8 Ma	✗	Fossil	POINAR et al. 2000
Simuliidae	Log Normal	163.5–174.1 Ma	✓	Fossil	CROSSKEY 1990
<i>Thaumalea testacea</i>	Uniform	Sc.1: 0–0.0135 Ma Sc.2: 0.117–0.128 Ma Sc.3: 1.4–1.8 Ma Sc.4: 0–2.588 Ma	✓	geologically estimated	EHLERS 2004 EHLERS 2004 EHLERS 2004 LOURENS et al. 2004
<i>Thaumalea bezzii</i>	Uniform	Sc.1: 0–0.0135 Ma Sc.2: 0.117–0.128 Ma Sc.3: 1.4–1.8 Ma Sc.4: 0–2.588 Ma	✓	geologically estimated	EHLERS 2004 EHLERS 2004 EHLERS 2004 LOURSENS et al. 2004
<i>Thaumalea verralli</i>	Uniform	0–0.0117 Ma	✓	geologically estimated	OLSEN et al. 2010
<i>Thaumalea truncata</i>	Uniform	Sc.1: 0–0.0135 Ma Sc.2: 0.117–0.128 Ma Sc.3: 1.4–1.8 Ma Sc.4: 0–2.588 Ma	✓	geologically estimated	EHLERS 2004 EHLERS 2004 EHLERS 2004 LOURENS et al. 2004
<i>Androposopa</i> gr. <i>larvata</i>	Uniform	Sc.1: 0–0.0135 Ma Sc.2: 0.117–0.128 Ma Sc.3: 1.4–1.8 Ma Sc.4: 0–2.588 Ma	✓	geologically estimated	EHLERS 2004 EHLERS 2004 EHLERS 2004 LOURENS et al. 2004

### 2.3. Population genetic analyses

Nucleotide diversity, SNP haplotype frequencies and pairwise distances within and among populations of *Androposopa* gr. *larvata*, *Thaumalea testacea* and *T. bezzii* were calculated using ARLEQUIN 2.0 (SCHNEIDER et al. 2000). Analysis of molecular variance (AMOVA, EXCOFFIER et al. 2005) was performed for species distributions between alpine and non-alpine region for *A. gr. larvata*. We used 10.000 permutations to test statistically significant fixation. Pairwise  $F_{ST}$  were calculated to test differentiation among populations using ARLEQUIN 2.0 (SCHNEIDER et al. 2000).

For *Thaumalea testacea* and *T. bezzii*, both with more than two population available, haplotype maps were generated using each locus both separately and concatenated. All haplotype maps were displayed with average uncorrected pairwise distances between populations and geographic distribution of haplotypes.

### 2.4. Phylogenetic inference

Substitution models for CO1 and WG data sets were estimated with MRMODELTEST2 (NYLANDER 2004) determining GTR+G+I Model for CO1 and GTR+G Model for WG as adequate. GARLI (ZWICKL 2006) was used to calculate a maximum likelihood phylogeny based on combined loci which was used as a reference for a Bayesian estimation of posterior probabilities using MrBayes 3.0 (HUELSENBECK & RONQUIST 2001). Substitution models for CO1 and WG data sets were estimated with MRMODELTEST2 (NYLANDER 2004). Markov chain Monte Carlo (B/MCMC) sampling consisted of two simultane-

ous chains for 10 million generations and a subsampling of every 100<sup>th</sup> tree for a total of 100.000 trees. Likelihood scores of samples were plotted against coefficient of variation using TRACER 1.6 ([http://evolve.zoo.ox.ac.uk/...](http://evolve.zoo.ox.ac.uk/)) to verify stationarity of the results (HUELSENBECK & RONQUIST 2011).

Phylogenetic trees were drawn in TREEVIEW 1.6.6 (PAGE 1996), and graphically improved with *Inkscape* (HARRINGTON et al. 2004, 2005).

Estimation of divergence times was assessed using BEAST v.1.8 (DRUMMOND et al. 2012) for combined CO1 and WG loci from 63 sequences. We used the substitution models GTR+G and GTR+G+I as recommended by our MrModeltest results. For the clock model, a log normal relaxed clock was assumed (DRUMMOND et al. 2006), using fossil calibration points for the basal most nodes and four different intraspecific diversification scenarios for intraspecific branching points. The different sets of priors are listed in Table 1. Due to our lack of fossil calibration points within Thaumaleidae, however, our divergence time analyses must be treated as very preliminary.

To investigate different diversification scenarios, four possible timeframes were chosen: (1) the last 1500 years of the late Pleistocene and Holocene (13.500 years until present); (2) the Riss-Würm interglacial starting approximately 128.000 years ago and ending 117.000 years ago; (3) the first half of the Biber-Donau interglacial ending with the Eburonium dating 1.8–1.4 Ma ago (EHLERS 2011); and (4) all interglacial periods from the onset of the Pleistocene 2.588 Ma ago to the present (LOURSENS et al. 2004). Scenarios 2–4 assume that speciation occurred prior to the last glaciation in Europe, with different species lineages presumably surviving in refugial zones in Ponto-Mediterranean provinces or near Alpine, Pyrenean

**Table 2.** FST-values and mean corrected pairwise distances between populations for *Thaumalea testacea* (T), *T. bezzii* (B) and *Androposopa gr. larvata* (A), estimated using Arlequin v2.0 with 10.000 permutations. Significant values are displayed in bold.

T	Gesäuse National Park	Thuringian Forest	Black Forest	Cochem-Zell	B	Gesäuse National Park	Thuringian Forest	Black Forest	Cochem-Zell	A	Gesäuse National Park	Rhön
Gesäuse National Park	*	0.84025	0.89189	0.84699	Gesäuse National Park	*	0.93651	0.95707	0.96748	Gesäuse National Park	*	0.78718
Thuringian Forest	<b>0.09863</b> ±0.0070	*	0.88539	0.80000	Thuringian Forest	0.99099 ±0.0030	*	0.93548	1.00000	Rhön	<b>0.01802</b> ±0.0121	*
Black Forest	<b>0.01953</b> ±0.0043	0.04688 ±0.0061	*	0.94737	Black Forest	0.09009 ±0.0235	0.33333 ±0.0273	*	0.89474			
Cochem-Zell	0.99902 ±0.0002	0.99902 ±0.0002	0.99902 ±0.0002	*	Cochem-Zell	0.99099 ±0.0030	0.99099 ±0.0030	0.99099 ±0.0030	*			

**Table 3.** Results of AMOVA for *Androposopa gr. larvata* based on 10.000 permutations. Va = Variation among groups, Vb = variation among populations within groups.

AMOVA			
Source of Variation	Sum of squares	Variance components	Percentage of Variation
Among populations	19.556	4.57937 Va	78.72
Within populations	8.667	1.23810 Vb	21.28
Total	28.222	5.81746	

or Carpathian mountain ranges. In the case of *T. verralli*, which populations were sampled from Norway and the Faroe Islands, only a Holocene timeframe was chosen because the Faroe Islands were covered with ice until only 11.700 years ago (OLSEN et al. 2010). Estimations were carried out by setting age priors for within species divergences according to the four scenarios. Some older fossil calibration points were also used in the analysis (Table 1).

Outgroup taxa were chosen based on published phylogenies on Culicomorpha (SAETHER 2000; BORKENT 2014) and the availability of fossil data. As a tree prior, we chose the Yule process model of speciation, the appropriate model assuming a pure birth process in which at any point in time, every lineage might undergo speciation or become extinct (GERNHARD 2008). The Bayesian parameters were set to a chain length of 10.000.000 and a log parameter of every 1000<sup>th</sup>.

Coalescent species delimitation was carried out using the Bayesian Poisson tree process (bPTP) as implemented by the bPTP web server (ZHANG et al. 2013), on the basis of a Co1 data set analysed in MrBayes 3.0 using 10 million MCMC generations and a burn-in of 0.2. For a discussion of species concepts and coalescent species delimitation, see FUJITA et al. (2012). Note that our use of coalescent methods in our analyses is only exploratory and we do not necessarily equate coalescence delimited species with species in the taxonomic sense.

Reconstruction of the ancestral area for *T. testacea* and *T. bezzii* was estimated with BEAST v.2.2 (DRUMMOND et al. 2012). The substitution model and MCMC preferences were set equal to divergence time estima-

tion, without setting any priors except the calibrated Yule model (HELED & DRUMMOND 2011). We chose this prior based on the multi-species dataset and assumed a constant lineage birth rate for each branch in the tree. The resulting file was imported into SPREAD v.1.0.6 (BIELEJEC et al. 2011) and analyzed using geographic coordinates. The resulting KML file was visualized using Google Earth (Google Inc.: Google Earth).

### 3. Results

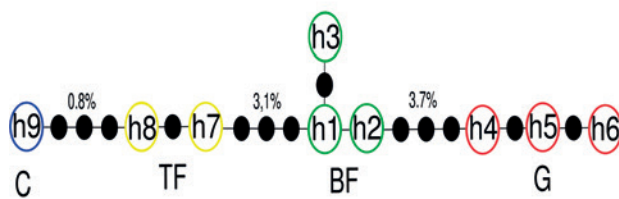
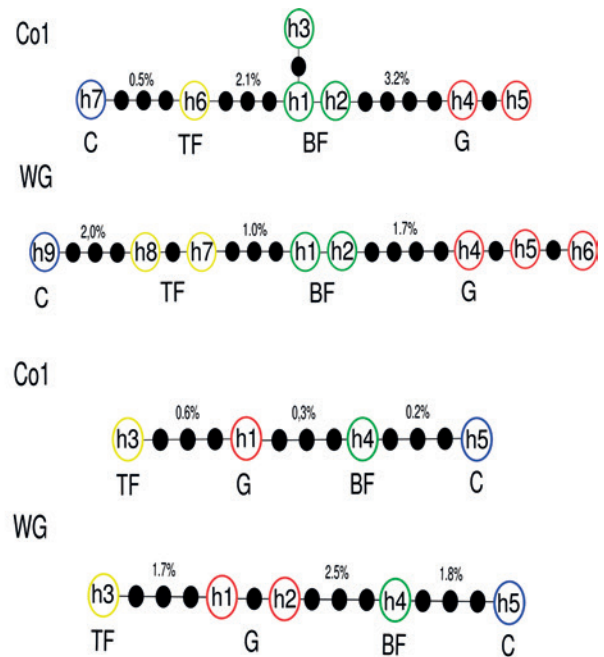
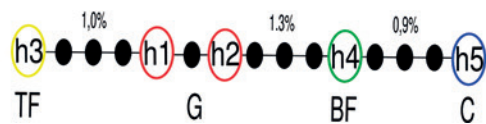
MtCo1 and WG sequences from 56 Thaumaleidae specimens were aligned into 658 bp (Co1) and 563 bp (WG) alignments, using sequences with no ambiguities. The Co1 alignment showed a strong bias towards A/T content: A: 26.6%; C: 18.2%; G: 17.8%; T: 3.5%. The WG alignment showed a balanced ratio between all bases: A: 24.0%; C: 22.2%; G: 23.1%; T: 30.7%. GenBank Accession numbers: KT215906–KT215962 for Co1 and KT215963–KT216019 for WG (see Supplementary Table S1).

#### 3.1. Population structure

*Thaumalea testacea* and *T. bezzii* showed a GTR+G (+I) corrected intraspecific distance in Co1 between haplotypes ranging from 0.5 to 3.2% for *T. testacea* and from 0.6 to 2.0% for *T. bezzii* (Fig. 1). GTR+G corrected distances for WG showed a 1.0–2.0% distance for *T. testacea* and 1.7–2.5% for *T. bezzii*. Based on strict molecular clock divergence rates for CO1 genes (BROWER 1994: Arthropod 2.3% / Ma; PAPADOPOULOU et al. 2010: 3.54% / Ma), all splits between populations within species in the data set indicate divergences during the Pleistocene.

All haplotypes were specific to collection sites and no shared haplotypes were detected.

Pairwise distances for Co1 in *T. testacea* are generally higher than in WG, while *T. bezzii* shows generally higher pairwise distances in the WG gene. The array of haplotypes for all populations regarding both species is mostly congruent (Fig. 1).

*T. testacea**T. bezzii*

**Fig. 1.** Haplotype distribution of *Thaumalea testacea* and *T. bezzii*. Concatenated loci (left) and single locus haplotype maps (right); groups are coloured according to locations. Percentages between locations are uncorrected pairwise distances. — **Abbreviations:** BF = Black Forest (green), Germany; G = Gesäuse National Park National Park, Austria (red); C = Cochem-Zell (blue), Germany; TF = Thuringian Forest, Germany (yellow).

Estimated  $F_{ST}$  values for *T. testacea*, *T. bezzii* and *A. gr. larvata* are displayed in Table 2. AMOVA results for *A. gr. larvata* (Table 3) indicate that most of the genetic variability occurs between populations (78.7%).

### 3.2. Phylogenetic inference

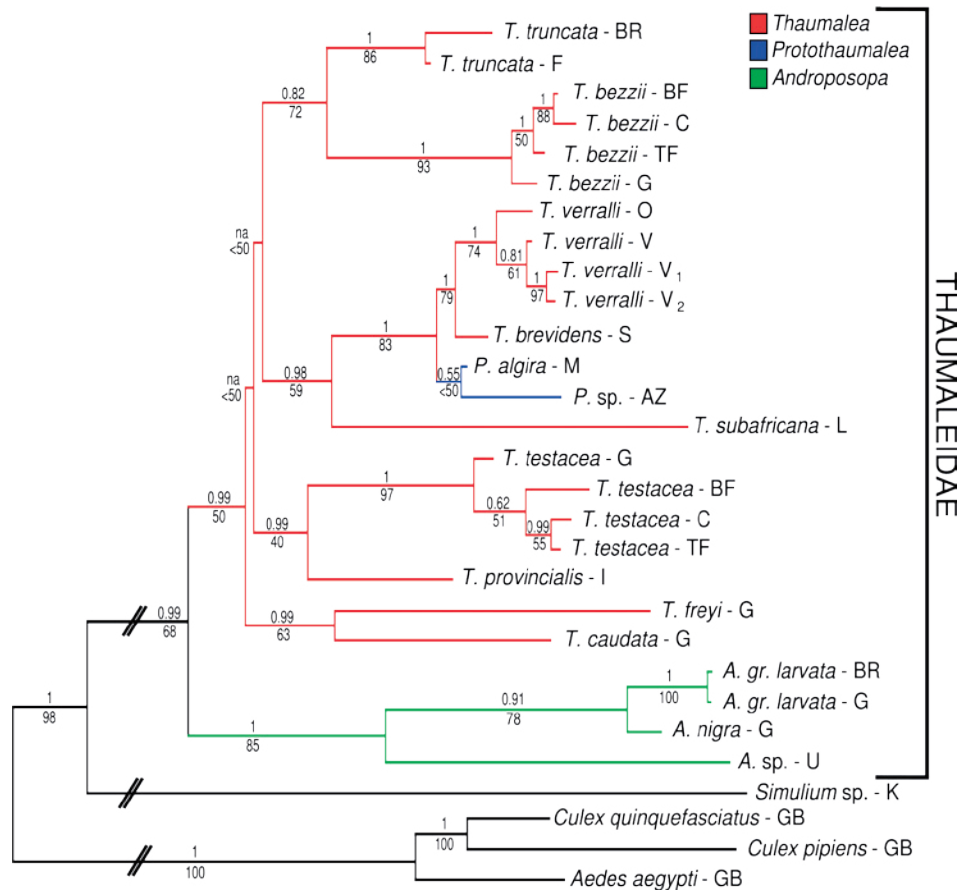
The topology estimated by Maximum Likelihood and Bayesian estimations was largely congruent (MrBayes and BEAST), (Fig. 2) and both bootstrap values and Bayesian posterior probabilities were generally high. *Androposopa* branches off as the sister group of a paraphyletic *Thaumalea* that includes *Protothaumalea* close to *T. verralli*. Species groups as postulated by WAGNER (2002) were not recovered.

The divergence of Thaumaleidae and Simuliidae was estimated in all scenarios to have occurred approximately 170 Ma ago (Figs. 3, S1–3). The split between the *Androposopa* and *Thaumalea* + *Protothaumalea* clades was estimated to occur 76–79 Ma ago, from the same period of the oldest known Thaumaleidae fossil (KOVALEK 1989). For scenario 1, the separation between all populations occurred all over the entire Holocene. For scenario 2, focusing on the Riss-Würm interglacial, all splits between populations tended to be as early as possible given previously set model boundaries after 0.12 Ma ago. Similar results were calculated for scenario 3, the Biber-Donau interglacial. When bounds were set to 1.8–1.4 Ma ago (EHLERS 2004), diversification among populations was estimated to have occurred 1.6–1.4 Ma ago. In the last scenario focusing on the entire Pleistocene (LOURSENS et

al. 2004), first separation of populations was displayed 2.3–2.2 Ma ago, followed by splits 1.6–1.2 Ma ago. For all scenarios, the colonization of the Faroe Islands by *T. veralli* was estimated to have occurred soon after the archipelago became ice free, about 11.700 years before present.

The bPTP analysis (Fig. 4) is consistent with all morphologically delimited species as treated by WAGNER (2002), and additionally suggested the existence of cryptic species within *T. testacea* and *T. truncata*. For *T. testacea*, three putative species were estimated within our data set; one distributed in Central Germany (Thuringian Forest and Cochem-Zell, support = 0.543) and one each in the Black Forest, Southern Germany (support = 0.558) and Gesäuse, Austria (support = 0.868). For *T. truncata*, the sampled populations from Finland and Germany were delimited as separate species with high support values of 0.999 (Finland) and 0.927 (Germany). However, as bPTP may be sensitive to incomplete or uneven sampling, these results should be interpreted with caution (ZHANG et al. 2013).

Ancestral area reconstruction estimated two different routes of immigration to Central Europe for *T. bezzii* and *T. testacea* from refugia in the Alps (Fig. 5). For *T. testacea*, estimations show a dispersal from the Alps to the Black Forest, proceeding further north to the Thuringian Forest and from there continuing to Cochem-Zell in northern Rhineland Palatinate. *T. bezzii* shows a comparable origin in the Alps, but dividing into the Thuringian Forest and the Black Forest populations, with the Rhineland Palatinate population branching off the latter.



**Fig. 2.** Unconstrained topologies based on maximum likelihood and Bayesian estimation. ML bootstrap values are plotted below and posterior probabilities above the branches. — **Abbreviations:** (1) Localities: AZ = Azila, Morocco; BF = Black Forest, Germany; BR = Biosphere Reserve Rhön, Germany; I = Civiasco, Italy; F = Enontekiö, Finland; G = Gesäuse National Park, Austria; U = Idaho, USA; K = Kassel, Germany; L = La Palma, Canary Islands, Spain; M<sub>1</sub> = Mallorca, Spain; N = NCBI Database; Ø = Øygarden, Norway; C = Cochem-Zell, Germany; S = Mangrt, Slovenia; TF = Thuringian Forest, Germany; V<sub>1</sub> = Viðoy, Faroe Islands; V<sub>2</sub> = Vágur 1, Faroe Islands; V<sub>3</sub> = Vágur 2, Faroe Islands. (2) Genera: A = *Androposopa*; T = *Thaumalea*; P = *Protothaumalea*.

## 4. Discussion

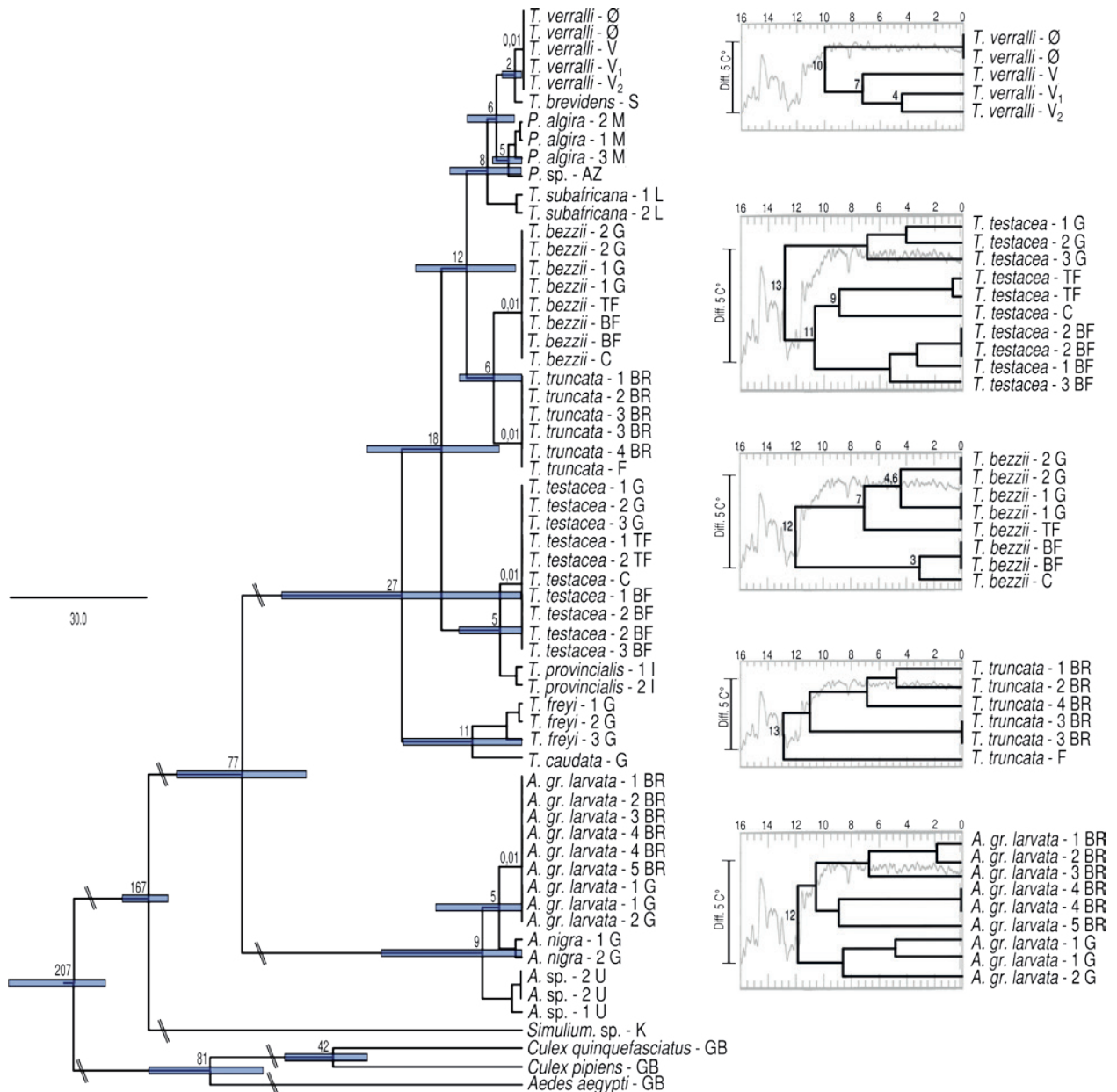
### 4.1. Systematic and taxonomic considerations

To the best of our knowledge, the present study is the first molecular phylogeny of Thaumaleidae, and, although the taxon sample is biased towards European fauna, some systematic and taxonomic conclusions can be drawn. The European Thaumaleidae fauna has recently been divided into two (VAILLANT & VINCON 1988; SINCLAIR 1996) or three (WAGNER 2002) genera, namely *Androposopa*, *Protothaumalea* and *Thaumalea*. Of these, VAILLANT & VINCON (1988) synonymized *Protothaumalea* with *Orphnephilina* (= *Androposopa*), without, however, mentioning specific characters to justify the synonymy. WAGNER (2002: p. 48) rejected the synonymy, separating the genera *Protothaumalea* and *Androposopa* by several characters of the male and female genitalia. Similarly, our results do not support the synonymy of *Protothaumalea* and *Androposopa*, as the two taxa do not form a monophyletic group in any of our analyses. On the oth-

er hand, we found *Protothaumalea* to be nested within *Thaumalea*, an unexpected relationship. Further study is necessary to assess whether there is any morphological character evidence to support this grouping.

The genus *Thaumalea* was provisionally divided by WAGNER (2002) into 13 species groups, based on differences in male genitalia. Our data set only includes members from seven of these groups, and for only two species groups do we have material from more than one species: *T. bezzii* and *T. testacea* (*T. testacea* group), and *T. truncata* and *T. provincialis* (*T. truncata* group). Both of these groups are non-monophyletic in our analyses, suggesting that the morphological characters used by WAGNER (2002) may not be reliable for phylogenetic inference.

EDWARDS (1928) and WAGNER (2002) considered *Androposopa larvata* to be a single, valid species but noted unusually high morphological variability among populations. According to WAGNER (2002), two distinct, geographically and ecologically separate morphotypes can be identified based on the degree of enlargement of the second palp segment. Whether these morphotypes are taxonomically recognizable entities depends on the considered species concept. In our data, only one population

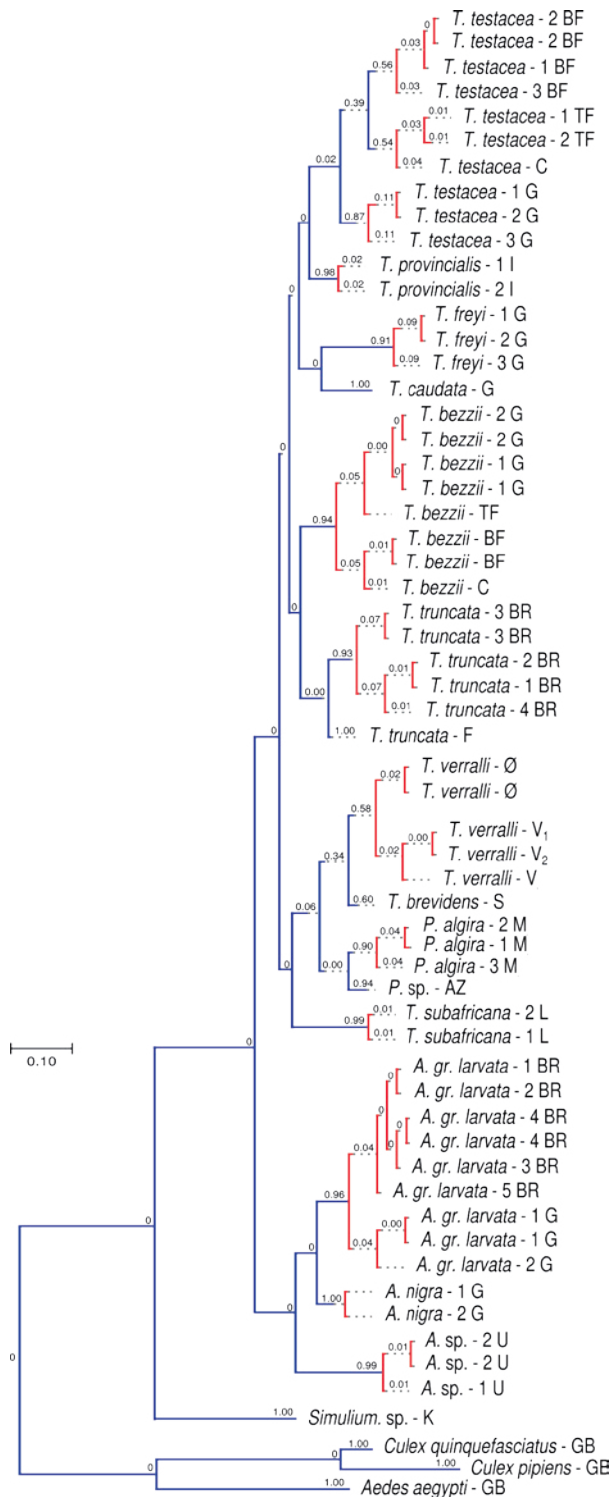


**Fig. 3.** Estimation of divergence times for scenario a (Holocene) using BEAST 1.8. The values represent estimated node ages. Blue bars indicate possible error frames. X-axis: substitutions per Site. Right: Species divergences mapped over temperature curve showing estimated times of dispersal between populations Temperature curves from SALTZMANN (2002). Axes: x = time in million years, y = July-temperature based on pollen in °C. Numbers before locality abbreviation indicate haplotype. — **Abbreviations: (1)** Localities: AZ = Azila, Morocco; BF = Black Forest, Germany; BR = Biosphere Reserve Rhön, Germany; I = Civiasco, Italy; F = Enontekiö, Finland; G = Gesäuse National Park National Park, Austria; U = Idaho, USA; K = Kassel, Germany; L = La Palma, Canary Islands, Spain; M<sub>1</sub> = Mallorca, Spain; GB = GenBank; Ø = Øygarden, Norway; C = Cochem-Zell, Germany; S = Mangrt, Slovenia; TF = Thuringian Forest, Germany; V<sub>1</sub> = Viðoy, Faroe Islands; V<sub>2</sub> = Vágar, Faroe Islands; V<sub>3</sub> = Vágar, Faroe Islands. **(2)** Genera: A = *Androposopa*; P = *Protothaumalea*; T = *Thaumalea*.

of each morphotype was available for study. However, these showed small but consistent genetic differences that explained 81% of the total genetic variation. This suggests that the populations have been separated for a long time. However, the bPTP analysis did not find any evidence for speciation, despite detecting putative species borders in the morphologically uniform species *T. testacea*. Based on the small number of specimens in the AMOVA, results should be interpreted with care, but we decided to include them nevertheless as they are potentially important for

future studies on Thaumaleidae. Furthermore, the differences between intra- and inter-population variation estimated by AMOVA are lower than typical threshold values for species delimitation (see e.g. HEBERT et al. 2003).

However, additional populations need to be sampled and both morphological and molecular characters should be considered in order to resolve the taxonomic status of *Androposopa larvata* in the sense of EDWARDS (1928) and WAGNER (2002), as well as the possible species borders with *A. rangifer* Martinovsky, 1999.



**Fig. 4.** Results of Poisson Tree Process (PTP) model to infer *Thaumalea* species boundaries on the Bayesian phylogenetic tree estimated by MrBayes. Red lines indicate delimited species. X-axis: substitutions per site. Numbers before locality abbreviation indicate haplotype. — **Abbreviations:** (1) Localities: AZ = Azila, Morocco; BF = Black Forest, Germany; BR = Biosphere Reserve Rhön, Germany; I = Civiasco, Italy; F = Enontekiö, Finland; G = Gesäuse National Park National Park, Austria; U = Idaho, USA; K = Kasel, Germany; L = La Palma, Canary Islands, Spain; M<sub>1</sub> = Mallorca, Spain; GB = GenBank; Ø = Øygarden, Norway; C = Cochem-Zell, Germany; S = Mangrt, Slovenia; TF = Thuringian Forest, Germany; V<sub>1</sub> = Viðoy, Faroe Islands; V<sub>2</sub> = Vágur, Faroe Islands; V<sub>3</sub> = Vágur, Faroe Islands. (2) Genera: A = *Androposopa*; P = *Protothaumalea*; T = *Thaumalea*.

This is also the case for *T. truncata* (= *T. cebennica* of WAGNER 2002, see ANDERSEN et al. 2013). Published illustrations of the internal genitalia of this species (TJEDER 1949; VAILLANT 1977; DISNEY 1999; WAGNER 2002; ANDERSEN et al. 2013) show small differences in the shape of the lateral ‘tooth’ of the paramere. The genetic distances between specimens from Finland and Germany are higher than those between *Androposopa* gr. *larvata* morphotypes, and bPTP results suggest them to be separate coalescent species with very high support. Thus, WAGNER’s (2002) delimitation of the *T. truncata* group into one North European and one Central European species may prove valid, even if it is partly based on incorrect taxonomic interpretations. DNA sequences of the species incorrectly treated as *T. truncata* by WAGNER (2002) are not available.

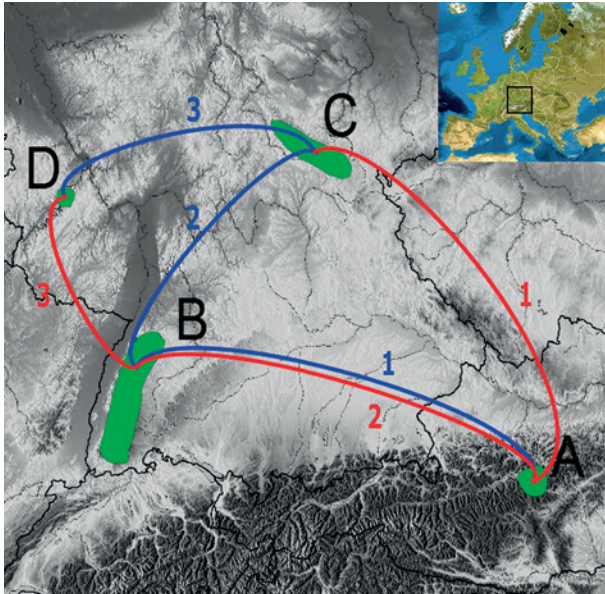
Conversely, no morphological variation corresponding to the genetic variability in *T. testacea* is known. Whereas the idea that *T. testacea* comprises several cryptic species cannot be rejected, it must be noted that bPTP support values for the “species” within this clade are low to moderate (0.54–0.87). In the absence of other lines of evidence, we interpret the perceived inter-population gaps in our data set as artifacts of uneven sampling in the phylogeny.

#### 4.2. Glacial dynamics and geography in the evolution of Thaumaleidae

Throughout the Pleistocene, isolation events may have occurred due to glacial events and changes in sea level that affected the landscape connectivity in Northern and Central Europe. In our analyses, we estimated population separations to have occurred either during the Holocene or during different intervals of the Pleistocene. All speciation scenarios resulted in almost identical phylogenies (Figs. 3, S1–3). In addition to maximum likelihood and Bayesian topologies, prior based estimation for species diversification calculated the split between *Androposopa* and *Thaumalea* to have occurred 76–79 Ma ago. In either scenario 2–4, the separation of populations for each species is calculated to have occurred in the beginning to the middle of prior set bounds. Based on strict molecular clock divergence rates for CO1 genes (BOWERS 1994: Arthropod 2.3% / Ma; PAPADOPOULOU et al. 2010: 3.54% / Ma), all splits between populations within species in the data set indicate divergences during the Pleistocene. The uncertainties are, however, high due to uneven temporal distribution of calibration points.

In general, the applied dating analysis is extremely preliminary due to a very patchy fossil record, which is why we used geological dates. Because of this lack of appropriate fossils, dating of internal nodes in the phylogeny is highly speculative. However, in all scenarios the majority of speciation events within each genus were estimated to have occurred between 4.8 and 32 Ma ago; i.e., the estimated speciation events fall within the Oligocene and Miocene periods. These periods are both char-





**Fig. 5.** Results of ancestral area reconstructions for *Thaumalea testacea* (blue) and *T. bezzii* (red) estimated by SPREAD using 10,000 permutations. Numbers before locality abbreviation indicate haplotype. — **Abbreviations:** A = Gesäuse National Park, Austria; B = Black Forest, Germany; C = Thuringian Forest, Germany; D = Cochem-Zell, Germany. Numbers indicate the chronological sequence of motion for *T. bezzii* and *T. testacea*.

acterized by aridity, in the Oligocene through increased glaciation and in the Miocene by formation of mountain chains (BEHRENSMEYER 1992; MILLER et al. 2006). For aquatic organisms, these climatic and geographical changes could have led to a decline of available habitats. Both this loss of habitat and the Miocene formation of mountains could fragment populations, possibly explaining speciation events in Thaumaleidae.

DOWNES (1988) hypothesized that the current insect fauna of the North Atlantic islands is the result of long-distance dispersal during the Holocene. This may explain the distribution of *T. verralli* which could have dispersed from Europe to the Faroe Islands and Iceland by air currents. Alternatively, its presence in Newfoundland on the east coast of Canada also could be the result of anthropogenic transport (SINCLAIR 1996). Our study suggests that *T. verralli* dispersed to the Faroe Islands by natural means rather than by human transport, as the divergence between the Norwegian and the Faroe populations is much older than the earliest evidence of human settlement on this peninsula (11700 b.p. rather than 1200 b.p., see HANNON et al. 2000).

Due to our small number of specimens, our ancestral area estimation is speculative. However, our preliminary analysis suggests that both *T. bezzii* and *T. testacea* migrated into northern Europe from different areas and followed different dispersal routes. These patterns correspond well with known habitat preferences and distributions (WAGNER 2002). *Thaumalea bezzii* is a lowland species, which has a southeastern distribution in Europe, and it has probably colonized Europe from a warm lowland glacial refugium in the Ponto-Mediterranean (WAGNER

2002). The comparatively low genetic distance between populations leads us to hypothesize that its presence in Northern Europe could be due to relatively recent dispersal events (possibly post-glacial dispersal). In contrast, *T. testacea* populations have been found to thrive at high altitudes in the Alps and in Corsica. The much higher interpopulation genetic distances in *T. testacea* could indicate that this species might have persisted in multiple refugia during the last ice age and the Holocene.

Despite its limitations, our data set supports the existence of multiple diversification patterns in Thaumaleidae, even if the different species have broadly similar life histories and habitat preferences. For cold-adapted species, the Alps could have served as a refugium in the Pleistocene whereas they functioned as stepping stones for more warm-adapted species in postglacial dispersal events. Alpine refugia can be considered an important sources of genetic variation for terrestrial species, suggesting that specialized spring organisms are not very different from other organisms in biogeographic patterns. However, as additional refugia could be found in other mountain ranges such as the Carpathian or Apuseni Mountains, our scenarios are likely to be at best highly simplified accounts of the lineages' biogeographical history (BOTOSANEANU 1975; DEFFONTAINE et al. 2005; SOMMER & BENECKE 2005; PAULS et al. 2009; URSENBACHER et al. 2006; BĂLINT et al. 2008; WAGNER 2002). Future studies should include more samples from Southern and Eastern Europe in order to further understand the biogeographic consequences of glaciation dynamics on spring-dwelling organisms.

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## Author contributions

This work is based on the master thesis work of Phillip Haubrock at the University of Kassel, Germany. Author contributions: R.W., H.L., P.H. & G.M.K. designed the study, R.W. and G.M.K. contributed specimens, R.W., P.H. and G.M.K. identified specimens, H.L. and P.H. developed lab protocols and performed lab work, P.H. and G.M.K. selected and performed data analyses, P.H. and G.M.K. wrote the manuscript, which was approved by all authors.

## Electronic Supplement Files

at <http://www.senckenberg.de/arthropod-systematics>

**File 1:** haubrock&al-thaumaleidae-asp2017-electronicsupplement-1.doc — **Table S1:** List and supplementary information of all specimens used in this study.

**File 2:** haubrock&al-thaumaleidae-asp2017-electronicsupplement-2.pdf — **Fig. S1.** Estimation of divergence times for scenario 2 (Riss-Würm interglacial) using BEAST 1.8. The values in the nodes represent ages. Blue bars indicate possible error frames. X-axis: substitutions per site. Right: Species divergences mapped over temperature curve showing estimated times of dispersal between populations. Temperature curves from GIBBARD & KOLFSCHOTEN (2004). Axes: x = time in million years, y = July-temperature based on pollen in °C. Numbers before locality abbreviation indicate haplotype. — **Fig. S2.** Estimation of divergence times for scenario 3 (Biber-Donau interglacial) using BEAST 1.8. The values in the nodes represent ages. Blue bars indicate possible error frames. X-axis: substitutions per site. Right: Species divergences mapped over temperature curve showing estimated times of dispersal between populations. Temperature curves from BERENDSEN (2004). Axes: x = time in million years, y = July-temperature based on pollen in °C. Numbers before locality abbreviation indicate haplotype. — **Fig. S3.** Estimation of divergence times for scenario 4 (Pleistocene) using BEAST 1.8. The values in the nodes represent ages. Blue bars indicate possible error frames. X-axis: substitutions per site. Right: Species divergences mapped over temperature curve showing estimated times of dispersal between populations. Temperature curves from BERENDSEN (2004). Axes: x = time in million years, y = July-temperature based on pollen in °C. Numbers before locality abbreviation indicate haplotype. — **Abbreviations:** (1) Localities: AZ = Azila, Morocco; BF = Black Forest, Germany; BR = Biosphere Reserve Rhön, Germany; I = Civiasco, Italy; F = Enontekiö, Finland; G = Gesäuse National Park National Park, Austria; U = Idaho, USA; K = Kassel, Germany; L = La Palma, Canary Islands, Spain; M<sub>1</sub> = Mallorca, Spain; GB = GenBank; Ø = Øygarden, Norway; C = Cochem-Zell, Germany; S = Mangrt, Slovenia; TF = Thuringian Forest, Germany, V<sub>1</sub> = Viðoy, Faroe Islands; V<sub>2</sub> = Vágur, Faroe Islands; V<sub>3</sub> = Vágur, Faroe Islands. (2) Genera: A = *Androposopa*; P = *Protothaumalea*; T = *Thaumalea*.

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